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ORIGINAL RESEARCH PAPER

MicroRNA-199-3p up-regulation enhances chondrocyte proliferation and inhibits apoptosis in knee osteoarthritis via DNMT3A repression

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Abstract

Aim Studies have pivoted on the position of microRNAs (miRNAs) in knee osteoarthritis (KOA) but not the more specific function of miR-199-3p. Thus, this study is to uncover the mechanism of miR-199-3p in KOA.

Methods Rats KOA models were established by modified Hulth method. miR-199-3p expression was observed in cartilage of KOA rats. The binding sites of miR-199-3p were predicted by bioinformatics analysis and the potential interaction between DNA methyltransferase 3A (DNMT3A) and miR-199-3p was verified by dual-luciferase reporter gene assay. Rats were injected with miR-199-3p agomir or antagomir and DNMT3A siRNA into the knee joint. Inflammatory response factors in serum and cartilage tissues, cell apoptosis, and pathological status of cartilage tissues were detected. Chondrocytes were isolated from KOA cartilages and treated with miR-199-3p mimic or inhibitor and DNMT3A siRNA. Chondrocyte proliferation and apoptosis were detected.

Results miR-199-3p expression was suppressed in cartilage of KOA rats. Dual-luciferase reporter gene assay proved that a miR-199-3p-binding site was located in the 3′UTR of DNMT3A mRNA. Inflammation, chondrocyte apoptosis and cartilage pathological changes were improved by miR-199-3p agomir but aggravated by miR-199-3p antagomir. The effects of miR-199-3p antagomir on KOA rats were partially reversed by DNMT3A siRNA. miR-199-3p mimic or DNMT3A siRNA decreased KOA chondrocytes apoptosis and promoted proliferation. miR-199-3p inhibitor showed the opposite functions to miR-199-3p mimic. The effects of miR-199-3p inhibitor on chondrocytes were reversed by DNMT3A siRNA.

Conclusion This study highlights that miR-199-3p up-regulation or down-regulation of DNMT3A induces chondrocyte proliferation and inhibits apoptosis in KOA, which may widen our eyes to treat patients with KOA.

Keywords Knee osteoarthritis · microRNA-199-3p · DNA methyltransferase 3A · Chondrocytes · Cartilage tissues · Proliferation · Apoptosis

Introduction

Knee osteoarthritis (KOA) is a chronic rheumatism characterized by progressive cartilage destruction which is mediated by cytokines and other molecules [1]. The continued development of KOA results in gradual impairment of knee function and causes pain and decreased muscle strength, thereby increasing the burden on the knee [2]. In fact, the risk factors for the onset of KOA consist of obese, history of knee injury, age and frequent kneeling and crouching [3]. Unfortunately, KOA is incurable except the treatment of complicated joint arthroplasty [4]. Thus, it is the priority to discover an innovative therapy to alleviate and even to cure KOA.

MicroRNAs (miRNAs) regulate varying cellular processes of which the deregulated expression is connected with

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OA [5]. Besides, miRNAs can meditate cartilage progression, homeostasis, and OA [6]. Moreover, previous studies have pivoted on different miRNAs to explore their specific roles in KOA with the conclusion that the expression of miR-140 and miR-130a is decreased in KOA [7, 8]. As we have known, miR-199a-3p is documented to participate in cell apoptosis in osteosarcoma with its reduced expression [9]. In addition, hsa-miR-199a-3p is reduced in stimulated human OA chondrocytes [10]. However, the uncovered role of miR-199-3p in KOA still needs exploration in depth. DNA methyltransferase 3A (DNMT3A), as a member of the DNA methyltransferase enzymes family, is inevitably involved in the catalysis of DNA methylation which is responsible for gene expression regulation and other crucial biological activities and processes [11]. Drawn from an advanced study, the underexpressed DNMT3A is found in OA cartilage tissues [11]. Surprisingly, the existed study has uncovered the relationship between DNMT3A and miR-199a-3p, suggesting that DNMT3A reduction markedly elevates the expression of miR-199a-3p [12].

Considering together, miR-199-3p and DNMT3A may be vital roles in KOA but without existed studies to uncover their combined interactions. Thus, this study is performed to uncover the concrete mechanisms of miR-199-3p and DNMT3A in KOA and to consolidate the therapeutic reference for KOA.

Materials and methods

Ethics statement

The animal experiment program was reviewed and approved by the ethics committee of The First Affiliated Hospital of Soochow University. Great efforts have been done to relieve the pain of animals.

Experimental animals

Sprague–Dawley 3-month-old rats of clean grade, half male and half female, weighing 250–300 g, were provided by the Experimental Animal Center of Soochow University (Suzhou, China). Rats were kept in a sterile cages, free to clean drinking water on commercial diet at 23–25 °C with relative humidity of 50% and 12 h light exposure.

Rat model establishment

Rat models of KOA were established using modified Hulth method [13]. Rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium at 30 mg/kg, fixed in a supine position with the right knee depilation and disinfection with 75% ethanol. The knee joint capsule was opened about 0.8 cm above the patella and went along with the medial edge of the patella to the tibial tuberosity. The tibial joint was dislocated in a straighten position and the knee joint was exposed in a flexed posture. The anterior ligament was severed, the medial meniscus was removed with the tibial joint reduction. The knee was processed with anterior drawer test and the hemostasis was performed in the operation area, the joint capsule and skin were sutured, and the operation area was re-sterilized with 75% ethanol. The knee in the sham group was only treated with joint capsule opening and suturing. The rats were not treated with injured knee fixation after operation and were free to move in cages. The operation was conducted in an aseptic environment.

Rats grouping

The rats were randomly divided into eight groups based on a table of random number, ten in each group including the sham group of rats only treated with joint capsule opening and suturing, the KOA group of rats with KOA model establishment, the agomir negative control (NC) group of rats with KOA model establishment and intra-articular injection of miR-199-3p agomir NC into the knee joint, the miR-199-3p agomir group of rats with KOA model establishment and intra-articular injection of miR-199-3p agomir into the knee joint, the si-NC group of rats with KOA model establishment and intra-articular injection of DNMT3A siRNA NC plasmids into the knee joint, the si-DNMT3A group of rats with KOA model establishment and intra-articular injection of DNMT3A siRNA plasmids into the knee joint, the miR-199-3p antagomir + si-NC group of rats with KOA model establishment and intra-articular injection of miR-199-3p antagomir and DNMT3A siRNA NC plasmids into the knee joint and the miR-199-3p antagomir + si-DNMT3A group of rats with KOA model establishment and intra-articular injection of miR-199-3p antagomir and DNMT3A siRNA plasmids into the knee joint. The agomir NC, miR-199-3p agomir or antagomir, si-NC, and si-DNMT3A plasmids were provided by Shanghai Genechem Co., Ltd. (Shanghai, China). Rats in interfere groups were injected with agomir NC, miR-199-3p agomir or antagomir, si-NC, and si-DNMT3A plasmids, respectively, at 60 mg/kg for 3 days after operation. Rats in the sham group and the KOA group were injected with normal saline at the same dose with the same frequency, injection method and location as the interfere groups. At the same time, mental status, dietary status, and joint redness and swelling of rats were observed.

Sample collection

Rats in each group were euthanized 2 weeks later since the last injection. Blood samples were obtained from the posterior venous plexus, sealed at 4 °C, centrifuged at 6000 r/

min with serum stored in eppendorf tubes at − 20 °C. The knee joint cartilage (the normal knee joint cartilage in the sham group and the cartilage of the injured part in other groups) were stored for tissue homogenate preparation. The right hind limbs were fixed with formalin and stored in 75% ethanol. The limbs were placed in 20% ethylenediaminetetraacetic acid solution, decalcified at 4 °C with the decalcified solution changed every 3 days. The limbs were dehydrated normally, embedded with paraffin and sectioned into 5-μm sections.

Pathological tissue section observation

Hematoxylin–eosin (HE) staining: Paraffin-embedded sections of cartilage tissue were dewaxed, dehydrated with gradient ethanol, stained with hematoxylin, differentiated with 1% hydrochloric acid, and treated with 1% ammonia. The tissues were then stained with 0.5% eosin, immersed in 95% ethanol, permeabilized with xylene and sealed in neutral gum. The morphology of the cartilage tissues was observed under an optical microscope.

Safranin O staining: Paraffin-embedded sections of cartilage tissues were dewaxed and stained with hematoxylin. Then tissues were differentiated with 1% hydrochloric acid ethanol, stained with 0.02% fast green solution and washed with 1% glacial acetic acid. Next, the tissues were stained with 0.1% Safranin O, washed with 95% ethanol, dehydrated with gradient ethanol, permeabilized with xylene and sealed in neutral gum. The morphology of cartilage tissues were observed under a light microscope. The cartilage tissue and cell structure, staining status, and tidal line were scored by the Mankin's method [14] with the higher score indicating the severer cartilage degeneration.

Toluidine blue staining: Paraffin-embedded sections of cartilage tissues were dewaxed, stained with 0.5% toluidine blue solution, differentiated with 0.5% glacial acetic acid solution, dehydrated with ethanol gradient, permeabilized with xylene and sealed in neutral gum. The digital medical image analysis system Med 6.0 was applied to analyze the average optical density (OD) of toluidine blue.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged at 8000 r/min with the supernatant stored at − 20 °C for detection. The contents of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and IL-6 in serum were detected by ELISA. The wells of the plastic plate coated with specific antibodies were added with 50 μL of serum and 50 μL of biotin-labeled secondary antibody, incubated and washed 3 times. Then the serum was added with 10 μL of streptavidin-labeled horseradish peroxidase, incubated and washed three times. Finally, 100 μL of buffer was added for reaction termination. The OD value was measured at 450 nm on a microplate reader. TNF-α, IL-6, IL-1β and ELISA kits were provided by NanJing JianCheng Bioengineering Institute (Nanjing, China).

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining

TUNEL staining was implemented to detect cell apoptosis in cartilage tissues with the instructions of the kit (Roche, USA). Paraffin sections were dewaxed, dehydrated and reacted with Protease K for 15–30 min. After that, the sections were cultivated with 50 μL TUNEL reaction solution for 1 h, 50 μL conversion agent pod for 30 min and 50–100 μL DAB for 10 min. Next, stained by hematoxylin, the sections were permeabilized by xylene, sealed and observed under five fields. TUNEL-positive rate was analyzed.

Chondrocyte primary culture and identification

The injured cartilage in the KOA group (the normal cartilage in the sham group) was excised into 2–3 mm under an aseptic condition, rinsed with phosphate buffered saline (PBS) to wash away the surface smudge and other tissues and cut into 1-mm^3 sections. Then the cartilage was centrifuged at 2000 r/min, detached with 0.25% trypsin, centrifuged again at 2000 r/min and added with 5 mL of 0.2% type II collagenase for detachment. Then, the cells were cultured in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) to remove the suspended cells. Upon 80% or above confluence, the cells were passaged by trypsinization. Chondrocytes were identified by toluidine blue staining and immunocytochemical staining of type II collagen.

Chondrocyte identification

Chondrocytes in the third passage were seeded into 24-well cell culture plates at 1×10^6 cells/mL, and covered with sterilized coverslips for incubation. Then, the medium was discarded and the cells were fixed with 4% paraformaldehyde and treated with 70% ethanol. Next, upon the ethanol removal, the cells were stained with toluidine blue solution containing 30% ethanol, rinsed with 100% ethanol, and sealed in neutral gum for observation and photographing under a light microscope.

Chondrocytes in the third passage were seeded into 24-well cell culture plates at 1×10^6 cells/mL, and covered with sterilized coverslips for incubation. Subsequently, the cells were fixed with 4% paraformaldehyde, incubated with freshly prepared 3% H_2O_2 and permeabilized with Triton X-100 on ice. Next, the cells were treated with

5 mg/mL bovine serum albumin and anti-type II collagen monoclonal antibody (1:100, Abcam Inc., Cambridge, MA, USA) as well as the biotin-labeled secondary antibody, and developed by avidin–biotin-peroxidase complex and diaminobenzidine. Finally, the cells were rinsed with sterile double distilled water, counterstained with hematoxylin, and sealed in neutral gum for observation under a light microscope.

Chondrocyte grouping and treatment

The cultured chondrocytes in the KOA group were divided into 7 groups including the KOA group of cells without transfection, the miR-199-3p mimic group of cells transfected with miR-199-3p mimic, the mimic NC group of cells transfected with mimic NC, the si-DNMT3A group of cells transfected with DNMT3A silencing vector, the si-NC group of cells transfected with DNMT3A silencing vector NC, the miR-199-3p inhibitor + si-DNMT3A group of cells transfected with miR-199-3p inhibitor and DNMT3A silencing vector and the miR-199-3p inhibitor + si-NC group of cells transfected with miR-199-3p inhibitor and DNMT3A silencing vector NC. A group with normal chondrocytes was set as the control group. mimic NC, miR-199-3p mimic or inhibitor, si-DNMT3A and its NC vectors were provided by GenePharma Co. Ltd. (Shanghai, China).

Cell counting kit-8 (CCK-8) assay

The cell proliferation was detected according to the instructions of the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Chondrocytes were seeded into 96-well plates at 5×10^3 cells/well in a mixture of 100 μL of DMEM (v/v, containing 10% FBS) and 10 μL of CCK-8 solution for 4-h incubation. The chondrocytes were cultured for 24, 48, and 72 h after transfection with three duplicates each group. The absorbance (A) value was measured at the wavelength of 450 nm by a microplate reader.

Flow cytometry

The chondrocytes were mixed with the culture medium. Then, the PBS-suspended chondrocytes were centrifuged at 1000 r/min three times, resuspended in 200 μL of binding buffer, and added with 10 μL of Annexin V-fluorescein isothiocyante (FIFC) and 10 μL of propidium iodide (PI). Finally, the chondrocytes were incubated without light exposure, added with 300 μL of binding buffer again, and detected by a flow cytometer with CXP analysis software.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cartilage tissues and chondrocytes using Trizol (Invitrogen, Carlsbad, California, USA) and transformed into cDNA via the cDNA Synthesis kit (Takara, Dalian, China). SYBR Green method was adopted to fluorescent quantitative PCR on ABI 7500. Primers (Table 1) were designed by Primer 6.0 software and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). U6 was applied as an internal control for miR-199-3p while β-actin for DNMT3A, TNF-α, IL-6 and IL-1β. The relative expression of target gene was calculated by $2^{-\Delta\Delta Ct}$ [15].

Western blot assay

Total protein was extracted from the knee joint cartilage tissues or chondrocytes, of which the concentration was measured by Braford assay (BioRad, Hercules, USA). Proteins were added with 5 × sodium dodecyl sulfate protein loading buffer (5:1) and boiled. The protein was transferred into polyvinylidene fluoride (PVDF) membrane, blocked with 5% skim milk powder and incubated with the primary antibodies DNMT3A (1:1000, ABI Company, Oyster Bay, NY, USA) and β-actin (1:2500, Wuhan Boster Biological Technology Co., Ltd., Hubei, China) as well as the corresponding secondary antibody (1:8000, Beyotime Biotechnology, Shanghai, China). The PVDF membrane was treated with enhanced chemiluminescence and analyzed by Image J software.

Table 1 Primer sequence

Gene	Primer sequence
miR-199-3p	Forward: 5′-GCGGCGGACAGTAGTCTGCAC-3’
	Reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3’
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3’
	Reverse: 5′-AACGCTTCACGAATTTGCGT-3’
DNMT3A	Forward: 5′-CATCTGCATCTCCTGTGGG-3’
	Reverse: 5′-GCAGTTTTGGCACATTCCTC-3’
TNF-α	Forward: 5′-CTCTTCTGCCTGCTGCACTTTG-3’
	Reverse: 5′-ATGGGCTACAGGCTTGTCACTC
IL-6	Forward: 5′-CCACTTCACAAGTCGGAGGCTTA-3’
	Reverse: 5′-GTGCATCATCGCTCTTCATACAATC-3’
IL-1β	Forward: 5′-AGGTCGTCATCATCCCACGAG-3’
	Reverse: 5′-GCTGTGGCAGCTACCTATGTCTTG-3’
β-actin	Forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3’
	Reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3’

miR-199-3p, microRNA-199-3p; DNMT3A, DNA methyltransferase 3A; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6

Dual luciferase reporter gene assay

The wild-type (WT) or mutant (MUT) 3′UTR of DNMT3A containing the putative binding sites of miR-199-3p was inserted into the pmiR-GLO vector, thereby pmir-GLO-DNMT3A-3′UTR WT and MUT were generated. DNMT3A-WT and DNMT3A-MUT vectors were constructed by GenePharma. pmir-GLO-DNMT3A-3′UTR WT or MUT, together with miR-199-3p mimic or mimic NC were transfected into chondrocytes through Lipofectamine 2000 (Invitrogen). Chondrocytes were transfected for 48 h and detected for luciferase activity via dual-luciferase reporter system (Promega, Madison, WI, USA).

Statistical analysis

Data analysis was performed using SPSS 21.0 statistical software (IBM, Chicago, IL, USA). Normality and homogeneity of variance tests were conducted. Data in normal distribution and homogeneity of variance were expressed as mean ± standard deviation, and compared by unpaired Student's *t* test (two groups) or one-way analysis of variance (ANOVA) (multiple groups) and post hoc tests. Otherwise, data were compared by rank sum test. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Decreased miR-199-3p and increased DNMT3A are expressed in cartilage tissues in KOA; miR-199-3p targets and inhibits DNMT3A expression

RT-qPCR and western blot analysis demonstrated that (Fig. 1a, b) the expression of miR-199-3p in the KOA group was decreased, while the expression of DNMT3A was increased in comparison to the sham group; in contrast to the agomir NC group, the expression of miR-199-3p was increased in the miR-199-3p agomir group and the expression of DNMT3A was decreased (both $P < 0.05$); by comparison to the si-NC group and the miR-199-3p antagomir + si-NC group, there was no significant difference in the expression of miR-199-3p in the si-DNMT3A group and the miR-199-3p antagomir + si-DNMT3A group (both $P > 0.05$) but the expression of DNMT3A was decreased (both $P < 0.05$). No significant discrepancy witnessed the expression of DNMT3A in the KOA group, the agomir NC group and the si-NC group (all $P > 0.05$).

To confirm that whether DNMT3A was targeted by miR-199-3p, TargetScan database analysis was employed to find that the binding site of miR-199-3p was located at the area from the 783 to 789 of the DNMT3A 3′UTR (Fig. 1c). The luciferase activity of cells co-transfected with DNMT3A-WT and miR-199-3p mimic was impaired, indicating that miR-199-3p specifically bound to DNMT3A (Fig. 1d).

Elevating miR-199-3p or knocking down DNMT3A improves cartilage pathological changes in KOA rats

Experimental rats were all survived without infection or death. Except for the sham group, the rats in other groups manifested by yellowish hair color with dullness and roughness. The knee joint activity of rats in the sham group was normal while that of rats in other groups was weakened.

HE staining demonstrated that the cartilage in the sham group was stained uniformly and darkly of which the overall structure was clear with smooth surface, basically normal and ordered chondrocytes, and complete but clear tidal line. In the KOA group, the agomir NC group and the si-NC group, the cartilage was thinned with faded staining, of which the overall structure was disordered with sunken cartilage surface, cartilage defect formation, unclear tidal line, visible fiber deformation and decreased chondrocytes. The cartilage in the miR-199-3p agomir group and the si-DNMT3A group was thinned and stained lightly with cartilage surface injury, increased chondrocytes with few chondrocyte hypertrophy and vacuolization. Most tidal line was complete, and some cartilage fibrosis was relieved versus their NC groups. The status of cartilage in the miR-199-3p antagomir + si-DNMT3A group was comparable to that in the KOA group, but better than that in the miR-199-3p antagomir + si-NC group (Fig. 2a).

Safranin O and fast green staining mirrored that the cartilage surface was smooth, matrix and chondrocytes were arranged in three specific regions and the whole layer was evenly dyed with red. The cartilage surface structure in the KOA group, the agomir NC group and the si-NC group was damaged with obvious cracks, lightly staining and destaining of cartilage and thinned cartilage. The miR-199-3p agomir group and the si-DNMT3A group demonstrated with uneven cartilage staining, intact tidal line and thick cartilage in comparison to their NC groups. The cartilage status in the miR-199-3p antagomir + si-DNMT3A group was comparable to that in the KOA group but better than that in the miR-199-3p antagomir + si-NC group (Fig. 2b).

Mankin's score comparison manifested that the KOA group acquired a high score in comparison to the sham group ($P < 0.05$). The scores in the KOA group, the agomir NC group and the si-NC group were not statistically significant (all $P > 0.05$). The scores were low in the miR-199-3p agomir group and the si-DNMT3A group were lower versus their NC groups (all $P < 0.05$). The score in the miR-199-3p antagomir + si-DNMT3A group was lower than that in the miR-199-3p antagomir + si-NC group ($P < 0.05$) (Fig. 2c).

Toluidine blue staining indicated that the cartilage was accompanied by intact surface and stained darkly and uniformly with the upper cartilage in blue–violet in the sham group. The KOA group, the agomir NC group and

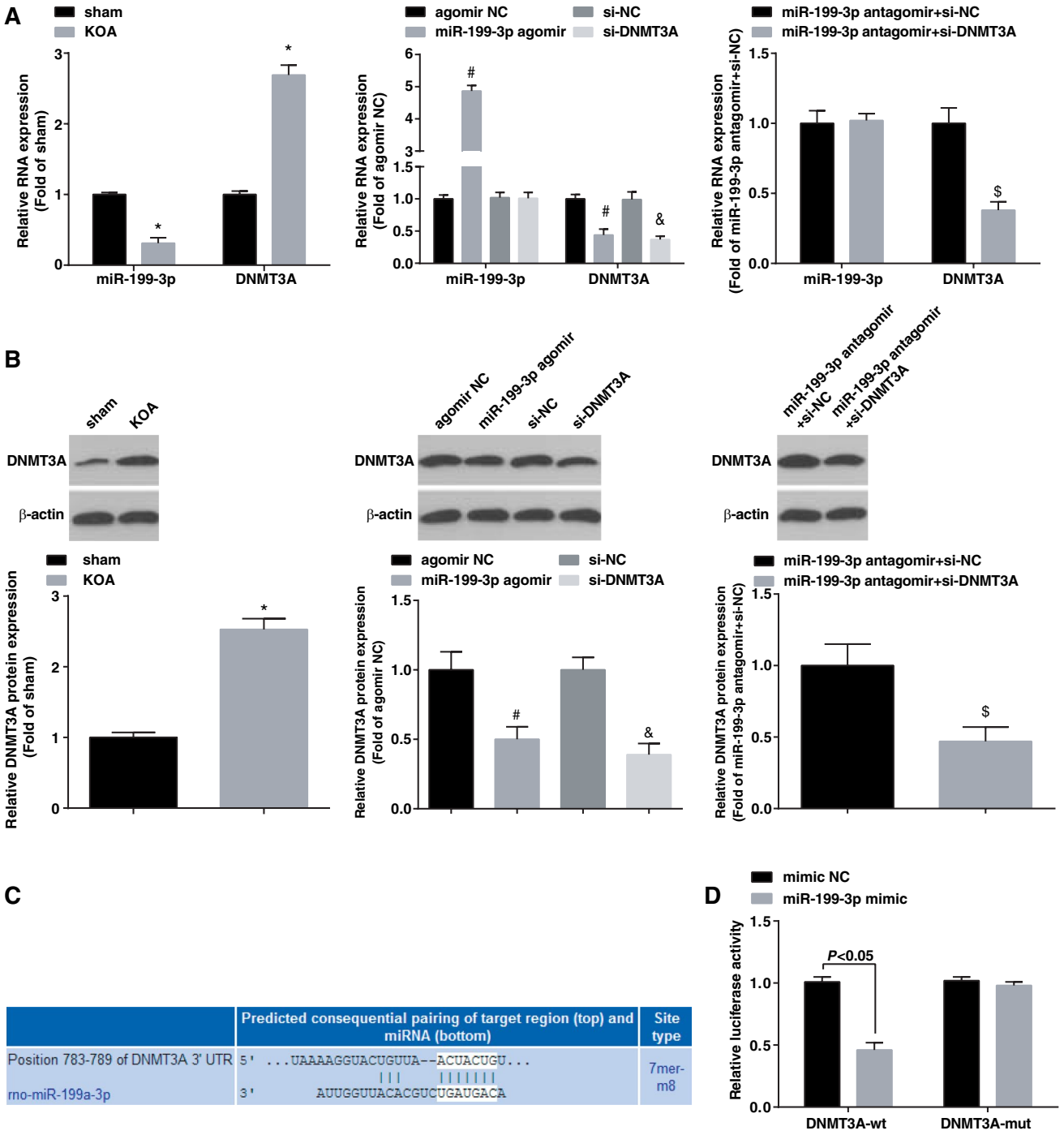

Fig. 1 Decreased miR-199-3p and increased DNMT3A are expressed in cartilage tissues in KOA rats; miR-199-3p targets and inhibits DNMT3A expression. **a** miR-199-3p and DNMT3A mRNA expression in cartilage tissues of each group detected by RT-qPCR; **b** DNMT3A protein expression in cartilage tissues of each group detected by western blot analysis; in figure **a/b**, $n = 10$; **c** The binding site of miR-199-3p and DNMT3A predicted by Targetscan; **d** Luciferase activity of each group; Repetitions = 3; $^*P < 0.05$ compared with the sham group; $\#P < 0.05$ compared with the agomir NC group; $\&P < 0.05$ compared with the si-NC group; $\$P < 0.05$ compared with the miR-199-3p antagomir + si-NC group. Data were expressed as mean ± standard deviation. Unpaired Student's *t*-test was used for comparison between the two groups, and one-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups

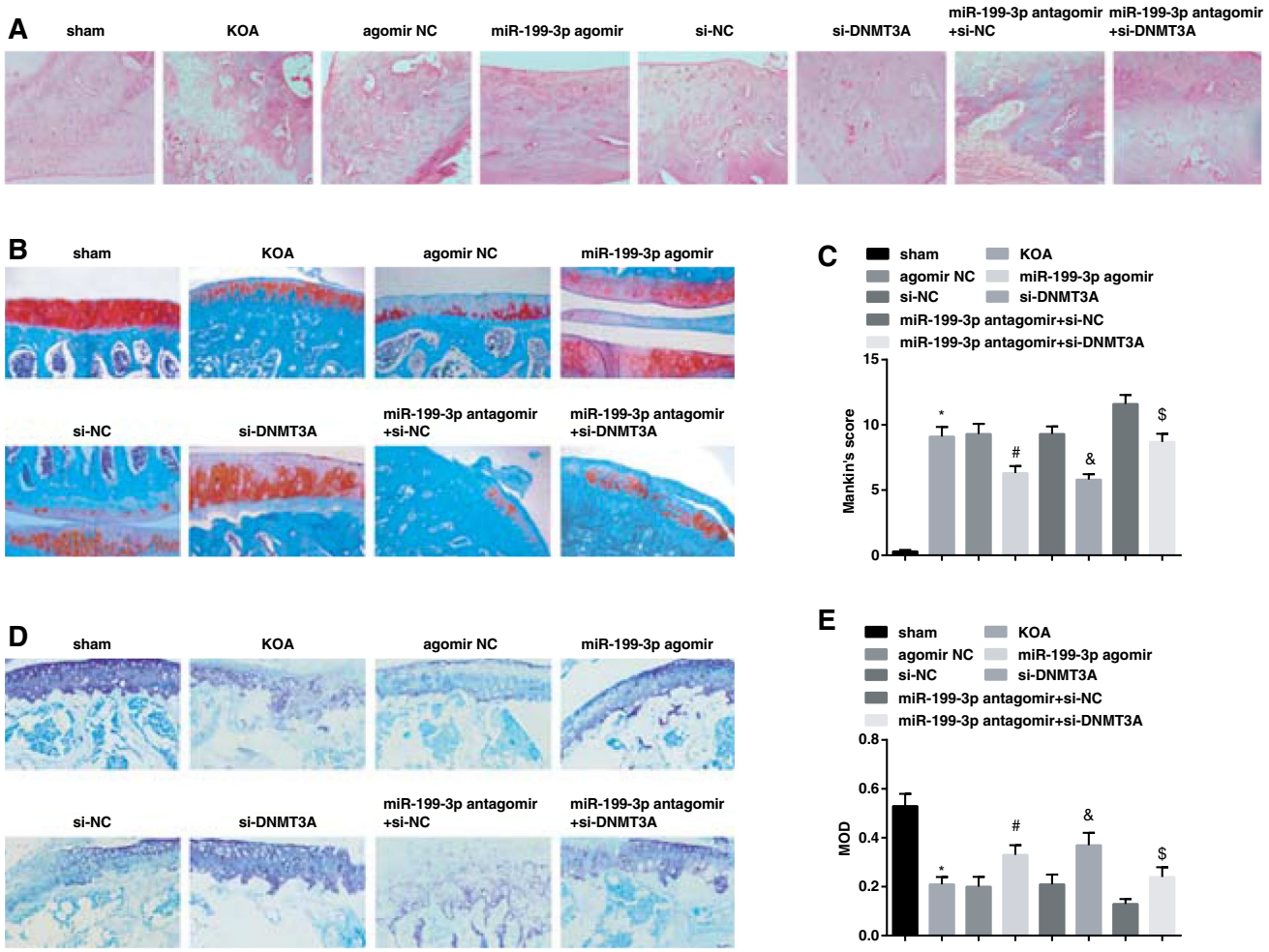

Fig. 2 Elevating miR-199-3p or knocking down DNMT3A improves cartilage pathological changes in KOA rats. **a** HE staining of knee cartilage tissues in each group (×200); **b** Safranin O and fast green staining of knee cartilage tissues in each group (×100); **c** Mankin's scores of knee joints in each group; **d** Toluidine blue staining of knee cartilage tissues in each group (×100); **e** Average optical density of knee cartilage tissues in each group after toluidine blue staining; $n=10$; $^*P<0.05$ compared with the sham group; $\#P<0.05$ compared with the agomir NC group; $\&P<0.05$ compared with the si-NC group; $\$P<0.05$ compared with the miR-199-3p antagomir + si-NC group. Data were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups

the si-NC group demonstrated with significantly reduced chondrocytes, cartilage exfoliation, subchondral bone exposure and cartilage destaining with cartilage in blue, indicating a decrease in proteoglycan content ($P<0.05$). The miR-199-3p agomir group and the si-DNMT3A group manifested with slightly decreased chondrocytes, and thinner cartilage layer. The cartilage was in blue-violet which was exactly between the manifested color in the sham group and their NC groups. The proteoglycan content was higher than that in the NC groups (both $P<0.05$). The toluidine blue staining in the miR-199-3p antagomir + si-DNMT3A group was comparable to that in the KOA group, and the cartilage color was darker than that in the miR-199-3p antagomir + si-NC group, indicating a higher proteoglycan content ($P<0.05$) (Fig. 2d, e).

Elevating miR-199-3p or knocking down DNMT3A attenuates inflammation reaction in KOA rats

ELISA assay and RT-qPCR elucidated that relative to the sham group, TNF-α, IL-6 and IL-1β levels in the KOA group in serum and cartilage tissues were increased (all $P<0.05$) while decreased in the miR-199-3p agomir group and the si-DNMT3A group in comparison to their NC groups (all $P<0.05$). TNF-α, IL-6 and IL-1β levels in the miR-199-3p antagomir + si-DNMT3A group were reduced versus the miR-199-3p antagomir + si-NC group ($P<0.05$). No conspicuous differences witnessed in the contents of TNF-α, IL-6 and IL-1β in serum and cartilage tissues in the KOA group, the agomir NC group and the si-NC group (all $P>0.05$) (Fig. 3a, b).

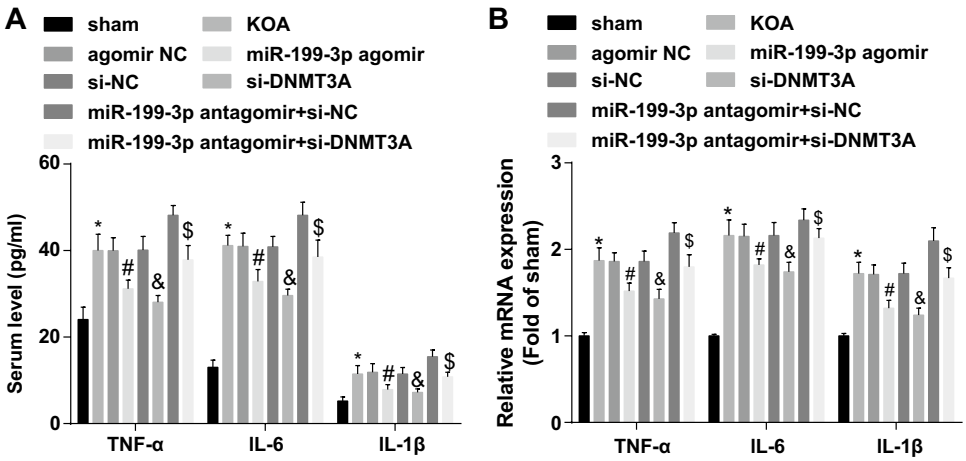

Fig. 3 Elevating miR-199-3p or knocking down DNMT3A attenuates inflammation in KOA rats. **a** TNF-α, IL-6 and IL-1β contents in serum of each group detected by ELISA; **b** TNF-α, IL-6 and IL-1β mRNA expression in cartilage of each group detected by RT-qPCR. $n=10$; $^*P<0.05$ compared with the sham group; $\#P<0.05$ compared with the agomir NC group; $\&P<0.05$ compared with the si-NC group; $\$P<0.05$ compared with the miR-199-3p antagomir + si-NC group. Data were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups

Up-regulation of miR-199-3p or down-regulation of DNMT3A inhibits apoptosis in cartilage tissues in KOA rats

TUNEL assay was employed to detect the number of apoptotic cells in cartilage tissues (Fig. 4a, b). It was elucidated that versus the sham group, the TUNEL-positive cells in the KOA group was increased ($P<0.05$), while it was decreased in the miR-199-3p agomir group and the si-DNMT3A group by comparison to their NC groups (all $P<0.05$). TUNEL-positive cells were lower in the miR-199-3p antagomir + si-DNMT3A group than the miR-199-3p antagomir + si-NC group ($P<0.05$). No significant differences existed in TUNEL-positive cells in the KOA group, the agomir NC group and the si-NC group (all $P>0.05$).

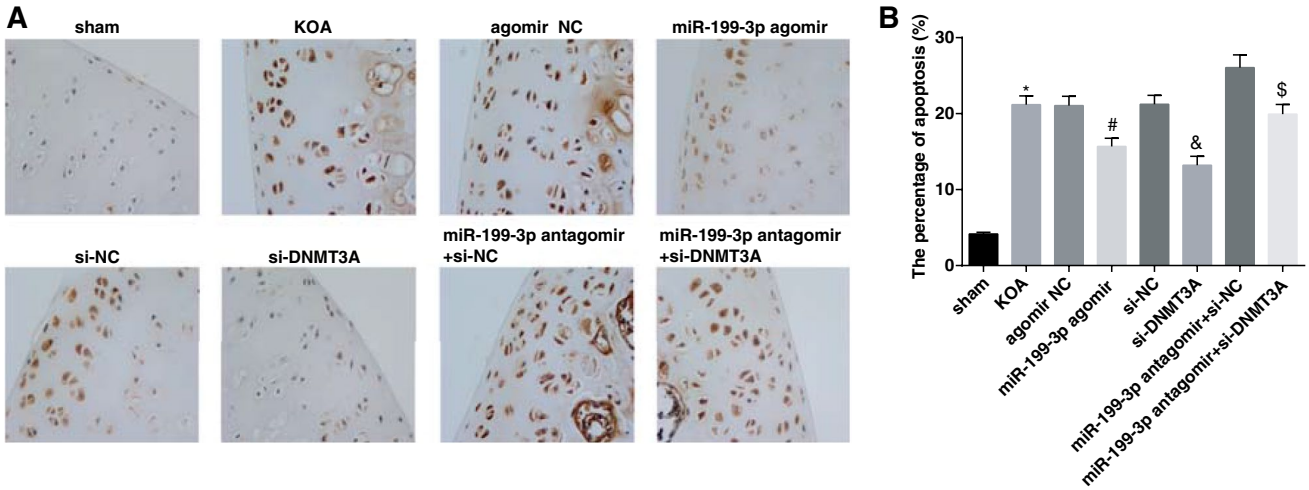

Fig. 4 Up-regulation of miR-199-3p or down-regulation of DNMT3A inhibits apoptosis in cartilage tissues in KOA rats. **a** TUNEL staining in cartilage tissues of each group (×200); **b** TUNEL-positive cells in cartilage tissues of each group; $n=10$; $^*P<0.05$ compared with the sham group; $\#P<0.05$ compared with the agomir NC group; $\&P<0.05$ compared with the si-NC group; $\$P<0.05$ compared with the miR-199-3p antagomir + si-NC group. Data were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups

Decreased miR-199-3p and increased DNMT3A are expressed in chondrocytes from KOA rats

Toluidine blue staining demonstrated that (Fig. 5a) the cultured cells grew with monolayer in the polygonal shape. The characteristic secretion proteoglycan was metachromatic with toluidine blue solution, with the cytoplasm in blue-violet and the nucleus in blue; immunocytochemical staining of collagen II revealed that (Fig. 5b) the cytoplasm was loose in pale yellow to brown, and the nucleus was large in the round or elliptical shape and located in the center of the cell. The cells were counterstained with hematoxylin in blue. The cultured cells were thus confirmed to be chondrocytes.

To validate the effects of miR-199-3p on DNMT3A, miR-199-3p and DNMT3A expression in chondrocytes were detected by RT-qPCR. The results elucidated that (Fig. 5c, d) relative to the control group, miR-199-3p expression was decreased and DNMT3A expression was increased in the KOA group (all $P < 0.05$); in contrast to the mimic NC group, the si-NC group and the miR-199-3p inhibitor + si-NC group, DNMT3A expression was decreased in the miR-199-3p mimic group, the si-DNMT3A group and the miR-199-3p inhibitor + si-DNMT3A group (all $P < 0.05$). miR-199-3p expression in the miR-199-3p mimic group was increased ($P < 0.05$), whereas it was not changed in the si-DNMT3A group ($P > 0.05$) in contrast to its NC group.

Up-regulation of miR-199-3p or down-regulation of DNMT3A enhances proliferation and inhibits apoptosis in chondrocytes from KOA rats

The chondrocyte proliferation after 24, 48 and 72 h of transfection was detected by CCK-8 assay. The results demonstrated that (Fig. 6a) versus the control group, the chondrocyte proliferation in the KOA group was impaired ($P < 0.05$). There was no significant discrepancy in chondrocyte proliferation among the KOA group, the mimic NC group and the si-NC group (all $P > 0.05$). The miR-199-3p mimic group and the si-DNMT3A group demonstrated with an enhancement in chondrocyte proliferation by comparison to their NC groups (all $P < 0.05$). The miR-199-3p inhibitor + si-DNMT3A group demonstrated with an enhancement in chondrocyte proliferation by comparison to miR-199-3p inhibitor + si-NC group ($P < 0.05$). It was indicated that up-regulation of miR-199-3p or down-regulation of DNMT3A could promote chondrocyte proliferation.

Flow cytometry was employed to detect chondrocyte apoptosis. The results elucidated that (Fig. 6b, c) by comparison to the control group, the apoptosis rate of chondrocytes in the KOA group was increased ($P < 0.05$). There was no discrepancy in the apoptosis rate of chondrocytes among the KOA group, the mimic NC group and the si-NC group (all $P > 0.05$). The apoptosis rate of chondrocytes in the miR-199-3p mimic group and the si-DNMT3A group was decreased in comparison to their respective NC groups (all $P < 0.05$). The miR-199-3p inhibitor + si-DNMT3A group showed lower apoptosis rate of chondrocytes versus the miR-199-3p inhibitor + si-NC group ($P < 0.05$).

Discussion

KOA is a degenerative joint disease of articular cartilage with its prevalence in the elderly [16]. miRNAs are convinced to be participators in cartilage development and arthritis pathogenesis, and especially, miR-199 are confirmed as chondrocyte-specific miRNAs [17]. However, almost no accurate description has depicted the mechanism of miR-199-3p in KOA. Considering that, this study is performed to decipher the uncovered mechanism of miR-199-3p in KOA, especially pivoting on the combined interaction of miR-199-3p and DNMT3A. Our study has expounded that up-regulation of miR-199-3p or down-regulation of DNMT3A enhances chondrocyte proliferation and inhibits apoptosis in KOA (Fig. 7).

The present study has elaborated that inflammatory response is enhanced in serum in KOA. An observational study has expounded that IL-1, IL-6 and TNF-α are critical mediators in pathogenic process of OA and specifically, the content of TNF-α in both serum and joint fluid is conspicuously increased along with deterioration of OA [18]. What is more, it has been documented that except the presence of elevated IL-1, IL-6 and TNF-α in OA, the overexpression of these three factors aggravates OA progression [19].

Besides that, the present study has been conducted with the outcome indicating that miR-199-3p up-regulation or DNMT3A down-regulation inhibits apoptosis and enhanced proliferation of chondrocytes in KOA. As to the pro-proliferation function, a former study has elucidated that elevation of miR-199b-5p enhances proliferation of pancreatic β-cells [20]. Moreover, an observational study has been conducted with the outcome elaborating that miR-199b-3p elevation suppresses the apoptosis of cerebral microvascular endothelial cells in ischemic stroke [21]. Furthermore, it is documented that miR-200b-3p induces chondrocyte growth and proliferation via DNMT3A inhibition in OA [11]. As demonstrated in the study, the decreased expression of anti-apoptosis protein Bcl-2 and the increased expression of pro-apoptosis protein Bax and cleaved caspase 3 are found in cartilage tissues and chondrocytes in KOA. It has been recorded that increased Bax mRNA as well as decreased Bcl-2 mRNA is observed in cartilage tissues in OA [22]. The increase in the expression of cleaved caspase 3 is reported to exist in osteonecrosis of the femoral head [23]. Anyway,

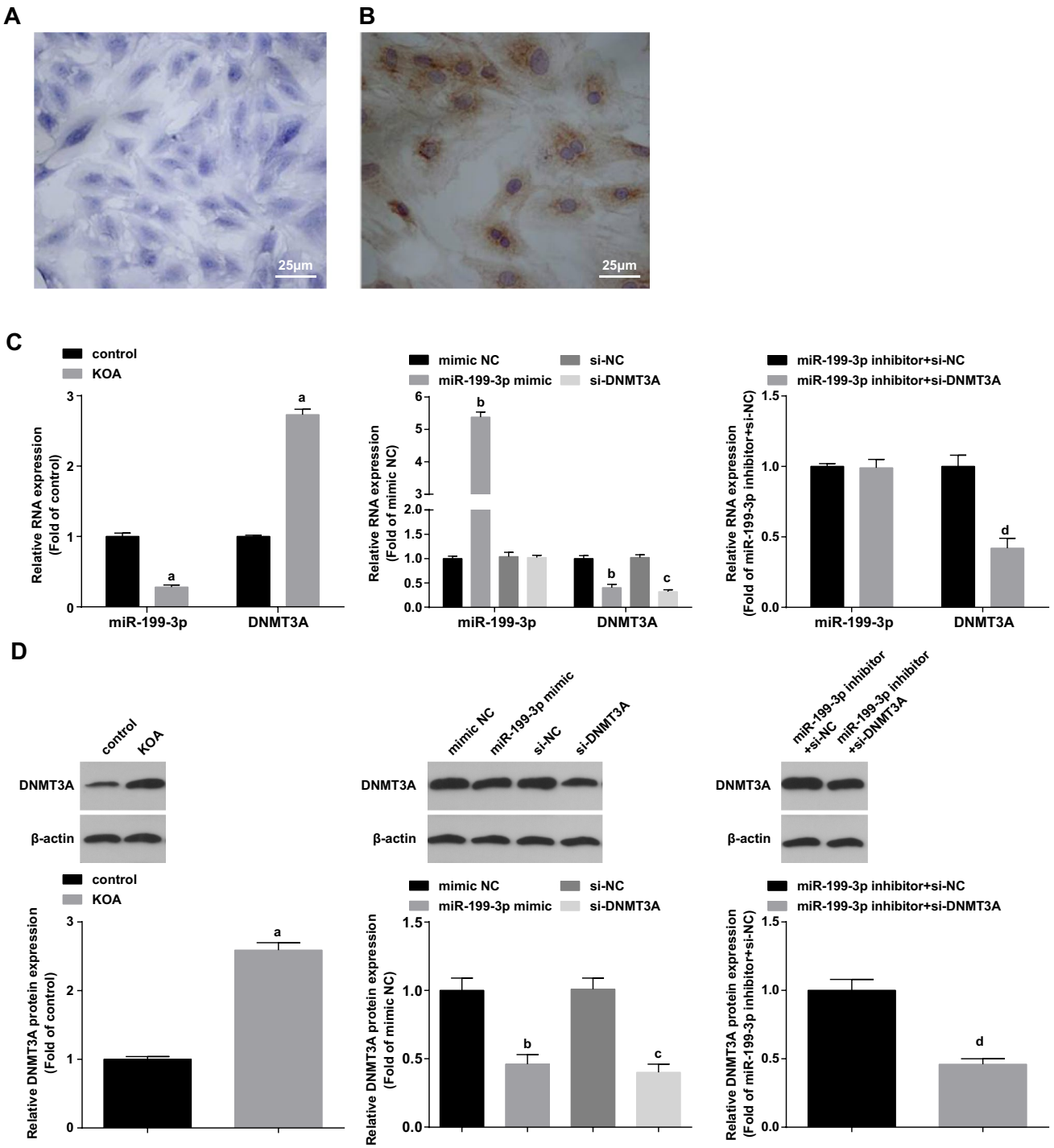

Fig. 5 Decreased miR-199-3p and increased DNMT3A are expressed in chondrocytes from KOA rats. **a** Toluidine blue staining of chondrocytes (400×); **b** Immunocytochemical staining of chondrocytes of collagen II (400×); **c** miR-199-3p and DNMT3A mRNA expression in chondrocytes of each group detected by RT-qPCR; **d** DNMT3A protein expression in chondrocytes of each group detected by western blot analysis; Repetitions = 3; a $P < 0.05$ compared with the control group; **b** $P < 0.05$ compared with the mimic NC group; **c** $P < 0.05$ compared with the si-NC group; **d** $P < 0.05$ compared with the miR-199-3p inhibitor + si-NC group. Unpaired Student's t-test was used for comparison between the two groups, and one-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups

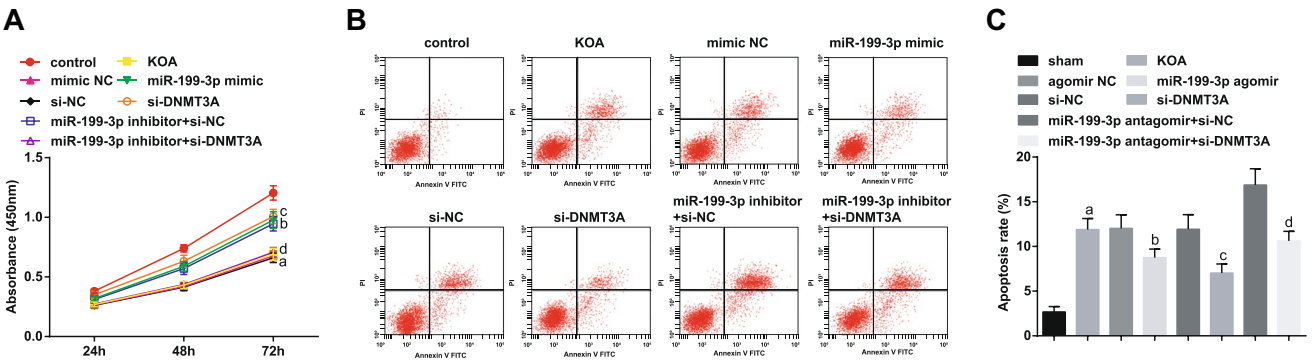

Fig. 6 Up-regulation of miR-199-3p or down-regulation of DNMT3A enhances chondrocyte proliferation and inhibits apoptosis in KOA. **a** Chondrocyte proliferation in each group detected by CCK-8 assay; **b** Apoptosis in each group detected by flow cytometry; C. Apoptosis rate in each group; Repetitions = 3; a $P < 0.05$ compared with the control group; **b** $P < 0.05$ compared with the mimic NC group; **c** $P < 0.05$ compared with the si-NC group; **d** $P < 0.05$ compared with the miR-199-3p inhibitor + si-NC group. Data were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups

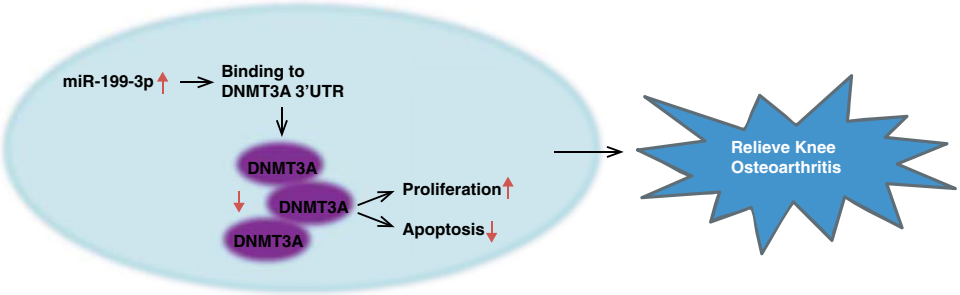

Fig. 7 The mechanism of miR-199-3p in knee osteoarthritis. DNMT3A is the target gene of miR-199-3p. Up-regulating miR-199-3p inhibits DNMT3A, thereby enhancing the proliferation ability and inhibiting the apoptosis of chondrocytes

these precedent studies are in conformity with our study outcomes.

In addition, we have concluded that the expression of miR-199-3p is underexpressed while DNMT3A is overexpressed in cartilage tissues and chondrocytes in KOA. To our best knowledge, miR-199a expression is proved to be reduced during BMP-2-induced chondrogenesis and blocks the approach to cartilage formation [6]. Moreover, it is found that the expression of miR-199a and miR-199b is reduced in human end-stage dilated cardiomyopathy [24]. The expression of DNMT3A has been surveyed with the manifestation of higher level in OA cartilage tissues [11]. It is documented that the activity of Adamts-5 is increased in articular chondrocytes in OA, which alters the physiological balance between matrix synthesis and degradation, thereby leading to enhanced proteolysis of Aggrecan [25]. According to a former study, the excessive degradation of collagen II and aggrecan commonly happens in the pathogenesis of OA [26]. In addition, our study has identified that miR-199-3p targets and inhibits DNMT3A expression. In fact, an existed study has already uncovered that DNMT3A is a direct target of miR-199a-3p [27].

In a word, this study has expounded the mechanism that up-regulation of miR-199-3 induces chondrocyte proliferation and suppresses apoptosis in KOA via DNMT3A repression. This study may update the clue for KOA treatment with miR-199-3/DNMT3A-based targeted therapy. Comprehensive and logical researches still should be performed to provide more curative options for patients with KOA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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